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Preclinical efficacy against toxic activities of medically relevant *Bothrops* sp. (Serpentes: Viperidae) snake venoms by a polyspecific antivenom produced in Mexico

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Abstract: The assessment of the preclinical neutralizing ability of antivenoms in Latin America is necessary to determine their scope of efficacy**.** This study was aimed at analyzing the neutralizing efficacy of a polyspecific bothropic-crotalic antivenom manufactured by BIRMEX in Mexico against lethal, hemorrhagic, defibrinogenating and *in vitro* coagulant activities of the venoms of *Bothrops jararaca* (Brazil), *B. atrox* (Perú and Colombia), *B. diporus* (Argentina), *B. mattogrossensis* (Bolivia), and *B. asper* (Costa Rica). Standard laboratory tests to determine these activities were used. In agreement with previous studies with bothropic antivenoms in Latin America, a pattern of cross-neutralization of heterologous venoms was observed. However, the antivenom had low neutralizing potency against defibrinogenating effect of the venoms of *B. atrox* (Colombia) and *B. asper* (Costa Rica), and failed to neutralize the *in vitro* coagulant activity of the venom of *B. asper* (Costa Rica) at the highest antivenom/venom ratio tested. It is concluded that, with the exception of coagulant and defibrinogenating activities of *B. asper* (Costa Rica) venom, this antivenom neutralizes toxic effects of various *Bothrops* sp venoms. Future studies are necessary to assess the efficacy of this antivenom against other viperid venoms. Rev. Biol. Trop. 65 (1): 345-350. Epub 2017 March 01.

Key words: snake venom, antivenom, *Bothrops*, *Crotalus*, neutralization.

The vast majority of snakebite envenomings occurring in Latin America are inflicted by species of the genus *Bothrops* (Fan & Cardoso, 1995; Warrell, 2004; Gutiérrez, 2010), which are distributed from Southern Mexico to Argentina (Campbell & Lamar, 2004). Depending on their severity, these envenomings are characterized by local tissue damage, i.e. edema, myonecrosis, hemorrhage, blistering, and by systemic alterations, i.e. hemorrhage, coagulopathies, acute kidney injury and cardiovascular shock (Warrell, 2004; Otero-Patiño, 2009; França & Málaque, 2009). Timely parenteral administration of antivenom is the only validated treatment for these envenomings. Diverse manufacturing laboratories in the region produce either monospecific or polyspecific bothropic antivenoms (Gutiérrez, Higashi, Wen, & Burnouf, 2007). The immunization mixtures used to generate these antivenoms greatly vary between laboratories. For instance, a polyspecific bothropic antivenom is manufactured by several laboratories in Brazil, using a mixture of the venoms of *Bothrops jararaca*, *B. jararacussu*, *B. moojeni*, *B. neuwiedii*, and *B. alternatus* as antigen (Cardoso, Yamaguchi, & Moura da Silva, 2009). On the other hand, a polyspecific antivenom produced in Costa Rica is generated by immunizing horses with a mixture of venoms of

Bothrops asper, *Crotalus simus* and *Lachesis stenophrys* (Segura et al., 2010).

There are situations in which antivenoms have to be imported from other countries in Latin America, either because there is no local production or because the national stocks of these immunobiologicals are insufficient. In these circumstances, it is highly relevant to ensure that antivenoms being imported are indeed effective in the neutralization of venoms of the most important snakes of the country. Hence, the preclinical assessment of the ability of antivenoms to neutralize venoms from species distributed in other countries in the region is important in order to have a knowledge-based platform for the distribution of antivenoms between countries in Latin America, often under the coordination of the Pan American Health Organization (PAHO) (Gutiérrez, 2014). Several studies have been performed in the past for assessing the preclinical efficacy of antivenoms in Latin America (see for example Otero et al., 1995; Gutiérrez, Rojas, Bogarín, & Lomonte, 1996; de Roodt, Dolab, Fernández, Segre, & Hajos, 1998; Bogarín et al., 2000). One of the most comprehensive analysis of the preclinical efficacy of antivenoms against venoms of various species of *Bothrops* sp. evaluated seven polyspecific antivenoms produced in Argentina, Brazil, Peru, Bolivia, Colombia and Costa Rica against the venoms of five species of *Bothrops* from different countries (Segura et al., 2010). This and other studies have underscored a widespread pattern of cross-neutralization of antivenoms against heterologous *Bothrops* sp. venoms. The present report extends these observations by investigating the preclinical efficacy of an antivenom manufactured in Mexico when confronted with the venoms of species of *Bothrops* from Brazil, Peru, Colombia, Argentina, Bolivia and Costa Rica.

MATERIALS AND METHODS

Venoms: The venoms of the following species were utilized: (a) *Bothrops diporus* (previously classified as *B. neuwiedi*) (Argentina), provided by Centro Nacional de Control de Calidad de Biológicos (CNCCB)-ANLIS "Dr Carlos G. Malbrán", Buenos Aires; (b) *B. mattogrossensis*) (previously classified as *B. neuwiedi*) (Bolivia), provided by the Instituto Nacional de Laboratorios de Salud (INLASA), La Paz; (c) *B. jararaca* (Brazil), provided by Instituto Butantan, Sao Paulo; (d) *B. atrox* (Peru), provided by Instituto Nacional de Salud (INS), Lima; (e) *B. atrox* (Colombia), provided by Instituto Nacional de Salud (INS), Bogotá; and (f) *B. asper* (Costa Rica), provided by Instituto Clodomiro Picado, Universidad de Costa Rica, San José. These venoms corresponded to the same pooled samples utilized in the study of Segura et al. (2010).

Antivenom: The antivenom tested is manufactured in Mexico by Laboratorios de Biológicos y Reactivos de México S.A. (BIRMEX; batch sv-162; in some experiments two additional batches were used: SV-165 and SV-187). This antivenom is produced from the plasma of horses immunized with a mixture of the venoms of *B. asper* and *Crotalus basiliscus* obtained from specimens collected in Mexico and kept at the serpentarium of BIRMEX. Fractionation of hyperimmune plasma is achieved by digestion of plasma proteins with pepsin at acid pH (Pope, 1939) and ammonium sulfate precipitation. It is a freeze-dried preparation composed of $F(ab')_2$ antibody fragments.

Neutralization of toxic activities: The following toxic activities of venoms were investigated: lethal (by the intraperitoneal route), hemorrhagic, defibrinogenating, and *in vitro* coagulant activities. The methods used for the characterization of the toxic activities of these venoms were described in a previous publication (Segura et al., 2010), and the same procedures were followed in the present work. For the analysis of the neutralization of these effects, a fixed amount of venom, which varies according to the effect to be studied, was incubated with various dilutions of the antivenom. Incubations were performed for 30 min at 37 ºC. Venoms were dissolved in 0.12 M NaCl, 0.04 M phosphate, pH 7.2 (PBS).

Controls included venom incubated with PBS without antivenom. After incubation, aliquots of the mixtures, containing a 'challenge dose' of venom, were tested in the corresponding assay systems described by Segura et al. (2010). The 'challenge doses' used were: For lethality, four Median Lethal Doses (LD_{50}s); for hemorrhage, five Minimum Hemorrhagic Doses (MHDs); for defibrinogenation, two Minimum Defibrinogenating Doses (MDDs); and for *in vitro* coagulation, two Minimum Coagulant Doses (MCDs). For lethal and hemorrhagic effects, the neutralizing efficacy was expressed as Median Effective Dose (ED_{50}), i.e. the ratio µL antivenom/mg venom in which the effect of the venom alone was reduced by 50 % (Gutiérrez et al., 1990). In the case of neutralization of lethality, results were also expressed as the ratio mg venom/mL antivenom. ED_{50} for lethality was estimated by probits (Finney, 1971). For defibrinogenating and *in vitro* coagulant activities, neutralization was expressed as Effective Dose (ED), as defined by Gené, Roy, Rojas, Gutiérrez and Cerdas (1989) and Segura et al. (2010).

RESULTS

The six venoms studied induced lethal, hemorrhagic, defibrinogenating and *in vitro* coagulant activities, as previously described (Segura et al., 2010). Since the venom pools used were the same as those utilized by Segura et al. (2010), the results of these toxicity tests are not reported here, and the reader is referred to this previous study. Regarding neutralization, the polyspecific antivenom of BIRMEX neutralized the lethal activity of all venoms tested, albeit with varying ED_{50}s depending on the venom (Table 1). Highest neutralization was achieved against the venoms of *B. diporus* (Argentina) and *B. mattogrossensis* (Bolivia), both of which were previously classified as *B. neuwiedi*, whereas the lowest neutralization was against the venom of *B. atrox* from Peru (Table 1). Antivenom was also effective in the neutralization of hemorrhagic activity of all venoms tested. Highest neutralization was achieved against the venoms of *B. asper* (Costa Rica) and *B. atrox* (Colombia), whereas the lowest neutralization was against the venom of *B. atrox* (Peru) (Table 1). Antivenom neutralized defibrinogenating activity of the venoms of *B. diporus* (Argentina), *B. atrox* (Peru), *B. mattogrossensis* (Bolivia) and *B. jararaca* (Brasil); in contrast, it required a high antivenom/venom ratio (4 000 µL antivenom/mg venom) to neutralize defibrinogenating activity of the venoms of *B. atrox* (Colombia) and *B. asper* (Costa Rica) (Table 1). Regarding *in vitro* coagulant activity, antivenom was effective in neutralizing the venoms of *B. atrox*

TABLE 1
Neutralization of toxic activities of *Bothrops* sp venoms by BIRMEX polyspecific antivenom[a]

Venom	Lethality (mg V/mL AV)[b]	Lethality (µL AV/mg V)[b]	Hemorrhagic (µL AV/mg V)	Defibrinogenating (µL AV/mg V)	Coagulant (µL AV/mg V)
B. atrox (Peru)	1.70 (1.28-2.16)	588 (463-781)	825 ± 93	2 000	425 ± 18
B. atrox (Colombia)	3.12 (2.31-4.22)	321 (237-433)	125 ± 18	4 000	1 096 ± 49
B. asper (Costa Rica)	2.34 (1.77-2.29)	427 (437-565)	136 ± 6	4 000	>4 000
B. diporus (Argentina)	6.75 (5.16-8.82)	148 (113-194)	151 ± 1	1 000	385 ± 7
B. jararaca (Brazil)	3.48 (2.36-4.62)	287 (216-424)	279 ± 12	1 000	438 ± 22
B. mattogrossensis (Bolivia)	4.44 (2.56-7.70)	225 (130-391)	210 ± 7	1 000	854 ± 35

[a]Neutralization of lethal, hemorrhagic and coagulant activities is expressed as Median Effective Dose (ED_{50}), whereas neutralization of defibrinogenating activity is expressed as Effective Dose (ED) (see the text for details). Results are presented as mean ± S.D. (n = 4), except in lethality where the 95 % confidence limits are included in parentheses.
[b]Neutralization of lethality is presented in two different ways: mg venom per mL antivenom, and µL antivenom per mg venom.

(Peru and Colombia), *B. diporus* (Argentina), *B. mattogrossensis* (Bolivia), and *B. jararaca* (Brasil), being ineffective, at the highest antivenom/venom ratio tested (4 000 µL antivenom/mg venom), to neutralize coagulant activity of the venom of *B. asper* from Costa Rica (Table 1). In order to corroborate this finding observed with the antivenom batch used (batch SV-162), two additional batches (SV-165 and SV-187) were tested for their ability to neutralize coagulant activity of Costa Rican *B. asper* venom. These two batches also failed to neutralize this effect at the highest antivenom/venom ratio tested.

DISCUSSION

Our results corroborate, for the polyspecific crotaline antivenom produced in Mexico by BIRMEX, the extensive cross-reactivity described for bothropic antivenoms in Latin America (Bogarín et al., 2000; Segura et al., 2010). In this case, when using the venoms of *B. asper* and *C. basiliscus* from Mexico in the immunizing mixture, the antivenom generated is effective for the neutralization of the four activities in the venoms of the South American species *B. jararaca*, *B. diporus*, *B. mattogrossensis* and *B. atrox* (Peru). The neutralization of lethality is the gold standard in the preclinical assessment of antivenom potency; in this regard, it is noteworthy that BIRMEX antivenom has a higher neutralizing ability of lethal effect of the venoms of *B. asper* (Mexico) (Segura et al., 2012), *B. diporus*, *B. jararaca*, *B. atrox* (Colombia), and *B. mattogrossensis* than against the venoms of *B. asper* (Costa Rica) and *B. atrox* (Peru). In contrast with other bothropic polyspecific antivenoms analyzed in previous works, BIRMEX antivenom has a relatively low neutralizing ability of defibrinogenating activity of the venoms of *B. asper* from Costa Rica and *B. atrox* from Colombia, and failed to neutralize *in vitro* coagulant activity of Costa Rican *B. asper* venom, at the highest antivenom/venom ratio used in this study (4 000 µL antivenom/mg venom).

A previous study showed that BIRMEX antivenom was highly effective in the neutralization of the venom of *B. asper* from Mexico, which is used in the immunizing mixture (Segura et al., 2012). The values of ED_{50} were 188 ± 27 µL antivenom/mg venom and 129 ± 4 µL antivenom/mg venom for lethal and hemorrhagic activities, respectively, and the values of ED were 1 135 ± 19 µL antivenom/mg venom and 2 000 µL antivenom/mg venom for *in vitro* coagulant and defibrinogenating actities, respectively (Segura et al., 2012). Moreover, in this study it was shown that BIRMEX antivenom was more effective in the neutralization of *in vitro* coagulant activity of the venom of *B. asper* from Mexico, as compared to the venom of *B. asper* from Costa Rica (Segura et al., 2012). This is likely to reveal differences in the immunological properties of procoagulant toxins in the venoms of *B. asper* from Mexico and Costa Rica, an issue that deserves further investigations. Owing to the relevance of coagulant and defibrinogenating activities in the overall pathophysiology of *Bothrops* sp. envenoming, these results suggest that BIRMEX antivenom may not be effective for the control of coagulopathies in envenomings by *B. asper* from Costa Rica, and may have a low efficacy for controlling defibrinogenation in envenomings by *B. atrox* from Colombia, probably requiring high volumes of antivenom to achieve therapeutic success. As a way to confront this problem, the venom mixtures used for horse immunization could be enriched with venoms of specimens of *B. asper* collected in countries other than Mexico.

In conclusion, the polyspecific bothropic-crotalic antivenom manufactured by BIRMEX in Mexico presents a pattern of cross-neutralization when confronted with heterologous *Bothrops* sp. venoms from various South American countries. However, this antivenom shows a low neutralizing ability against defibrinogenating and *in vitro* coagulant activities of the venom of *B. asper* from Costa Rica.

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RESUMEN

Eficacia de un antiveneno poliespecífico producido en México para neutralizar, a nivel preclínico, las actividades tóxicas de venenos de *Bothrops* sp (Serpentes: Viperidae) de importancia médica. Es necesario estudiar a nivel preclínico la capacidad neutralizante de los antivenenos producidos en América Latina, para conocer su espectro de cobertura. En este estudio se analizó la eficacia preclínica de un antiveneno poliespecífico botrópico-crotálico producido por BIRMEX, en México, para neutralizar los efectos letal, hemorrágico, desfibrinogenante y coagulante *in vitro* de los venenos de *Bothrops jararaca* (Brasil), *B. atrox* (Perú y Colombia), *B. diporus* (Argentina), *B. mattogrossensis* (Bolivia) y *B. asper* (Costa Rica). Se emplearon metodologías de laboratorio estándar en los análisis. En consonancia con estudios anteriores con diversos antivenenos botrópicos en América Latina, se observó un amplio patrón de neutralización de estos venenos heterólogos en la mayoría de los efectos estudiados. Sin embargo, el antiveneno mostró una baja capacidad neutralizante contra el efecto desfibrinogenante de los venenos de *B. atrox* (Colombia) y *B. asper* (Costa Rica) y no neutralizó la actividad coagulante *in vitro* del veneno de *B. asper* (Costa Rica) a la máxima razón antiveneno/veneno empleada.

Palabras clave: veneno de serpiente, antiveneno, *Bothrops*, *Crotalus*, neutralización.

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